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DOCUMENT-IDENTIFIER: US 6355418 B1
TITLE: Chimeric oligonucleotides and uses thereof in the identification of antisense binding sites

Abstract Text (1):
A chimaeric oligonucleotide library for use in identifying an antisense binding site in a target mRNA, comprising a plurality of distinct chimaeric oligonucleotides capable of hybridizing to mRNA to form a duplex, the nucleotide sequences of which each have a common length of 7 to 20 bases and are generated randomly or generated from information characterizing the sequence of the target mRNA, wherein substantially all the nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library, and wherein each nucleotide sequence comprises: a) a recognition region comprising a sequence of nucleotides which is recognizable by a duplex-cutting RNAase when hybridized to the mRNA, and b) a flanking region comprising a sequence of chemically-modified nucleotides which binds to the mRNA sufficiently tightly to stabilize the duplex for cutting of the mRNA in the duplex by the duplex-cutting RNAase, wherein the nucleotides constituting the flanking region are different from those constituting the recognition region, and wherein each oligonucleotide is protected against exonuclease attack.

Priority Application Year (1):
1995

Brief Summary Text (2):
The present invention relates to an oligonucleotide library, more particularly a chimaeric oligonucleotide library, and uses thereof in the identification of antisense binding sites in target mRNA and in providing potential therapeutic agents.

Brief Summary Text (4):
Antisense oligonucleotides are single-stranded nucleic acids which are complementary to the coding or "sense" strand of genetic material. An antisense oligonucleotide is therefore also complementary to the mRNA produced from the genetic material. If antisense DNA or RNA is present in a cell with the mRNA, hybridisation takes place to form a duplex thereby preventing translation of the mRNA by ribosomes to make a protein. Thus, antisense RNA can be used to block the expression of genes that make proteins.

Brief Summary Text (5):
The antisense approach to the inhibition of gene expression, though conceptually straightforward, presents technologically demanding challenges. A variety of approaches have been taken by various academic groups and biotechnology companies. Oligonucleotides have been made with sugar modifications, such as 2'-O allyl ribonucleotides, and with backbone modifications in the phosphate group, such as phosphorothioate deoxyribonucleotides. However, production of these individual oligonucleotides for application as antisense therapeutics, reagents or tools for drug target validation has been hampered because methods of identifying potentially efficacious antisense compounds against a given target mRNA are extremely difficult. Even with an mRNA of known sequence, it is often impossible to predict what sub-sequences in the target mRNA might be available for antisense binding because of the three-dimensional structure of the mRNA and the association of RNA with proteins.

Brief Summary Text (6):

In an alternative approach to the use of chemically modified oligonucleotides, Lieber and Strauss (ref 22) report the use of a ribozyme expression library for the purpose of selecting cleavage sites in target RNAs. The ribozyme approach suffers from the disadvantage that it requires cleavage sites containing GUC or CUC and thus is not generally applicable to all possible cleavage sites. In addition, cleavage efficiency is relatively low, and chemical synthesis of ribozyme libraries is difficult.

Brief Summary Text (8):
According to one aspect, the present invention provides a chimaeric oligonucleotide library for use in identifying an antisense binding site in a target mRNA, which comprises a plurality of distinct chimaeric oligonucleotides capable of hybridizing to mRNA to form a duplex, the nucleotide sequences of which each have a common length of 7 to 20 bases and are generated randomly or generated from information characterising the sequence of the target mRNA, wherein substantially all the nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library, and wherein each nucleotide sequence comprises:

Brief Summary Text (13):
Each chimaeric oligonucleotide forming the library may be made synthetically using any commonly-available oligonucleotide sequence synthesizer. The exact length of the nucleotide sequence will reflect a balance between achieving the necessary specificity and keeping the length to a minimum to minimise cost. Preferably, the nucleotide sequence has a length in the range 10 to 20, more preferably 14 to 17 bases, yet more preferably around 15 bases.

Brief Summary Text (26):
Oligonucleotide Libraries

Brief Summary Text (27):
In a further aspect, the present invention provides use of an oligonucleotide library in a method of identifying an antisense binding site in a target mRNA, wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases, preferably 10 to 20 bases, and each of which comprises a substrate for a duplex-cutting RNAase if hybridised to the mRNA, which library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library. The nucleotide sequences may comprise modified nucleotides, such as phosphorothioates, as described herein. The nucleotide sequences may be chimaeric or non-chimaeric.

Brief Summary Text (28):
In one embodiment, the library is generated randomly by means of an oligonucleotide sequence synthesizer. An aim of generating the sequences randomly is that substantially all possible nucleotide sequences of the specified length are generated. For a sequence of 10 bases in length (a 10-mer), 4.sup.10 distinct nucleotide sequences would need to be generated to cover all possibilities. This works out as approximately 10.sup.6 distinct nucleotide sequences. For a 15-mer, the library would need approximately 10.sup.9 to 10.sup.10 nucleotide sequences. Each nucleotide sequence will have a common length (i.e. they will all be 10-mers or will all be 11-mers, etc.). Any commonly-available oligonucleotide sequence synthesizer may be used for this purpose such as supplied by Applied Biosystems. All four possible bases are fed into the machine with an appropriate program using suitable nucleotides or modified nucleotides.

Brief Summary Text (29):
In an alternative embodiment, instead of generating the nucleotide sequences randomly they are generated from information characterising the sequence of the target mRNA. The sequence of the target mRNA needs to be known and can then be programmed into the oligonucleotide sequence synthesizer. For example, in the case of a gene which produces an mRNA of 450 nucleotides, a library of 15-mers would be produced with a total of 436 distinct nucleotide sequences (i.e. length of mRNA minus length of nucleotide sequence plus 1). In this way, all potential sub-sequences of the mRNA would be represented in the library. This is advantageous over the random generation of the library because there is no dilution of potentially useful nucleotide sequences

by randomly generated sequences not present in the target mRNA. A further way of ensuring that all sub-sequences of the mRNA are present in the library is to produce, in the case of an mRNA of 450 nucleotides, a library of 30 15-mers (i.e. length of mRNA divided by the length of the nucleotide sequence).

Brief Summary Text (31):
1) incubating with the target mRNA an oligonucleotide library and a duplex-cutting RNAase under conditions to produce target mRNA cut at the antisense binding site; and

Brief Summary Text (32):
2) identifying the antisense binding site from the position of the cut in the mRNA; wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases, preferably 10 to 20 bases, and each of which comprises a substrate for the duplex cutting RNAase if hybridized to the mRNA; and wherein the oligonucleotide library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of such common length which are present as sub-sequences in the target mRNA are present in the library.

Brief Summary Text (33):
Use of an oligonucleotide library in this manner enables identification of one or more antisense binding sites in a target mRNA and such identification can be achieved very rapidly in comparison with known methods. No information about the three-dimension structure of the mRNA is required because the identification of the antisense binding sites is empirical. Incubation of the target mRNA with the oligonucleotide library and the duplex-cutting RNAase can, by suitable variation of the reaction conditions, produce target mRNA cut at one or more antisense binding sites. This is because the library will contain one or more oligonucleotides which are complementary to such binding sites and will bind thereto under appropriate conditions to form a duplex. The duplex acts as a substrate for the duplex cutting RNAase. When the mRNA is cut at the binding site the oligonucleotide is released and is thereby made available for further binding. The oligonucleotide therefore acts catalytically and the duplex-cutting RNAase acts enzymatically. The duplex-cutting RNAase is separate from the oligonucleotide library and is preferably from a cell extract. Preferably, the duplex-cutting RNAase is RNAase H. The target mRNA is also preferably from a cell extract. Advantageously, therefore, both RNAase H and mRNA are present in the same cell extract with which the oligonucleotide library is incubated.

Brief Summary Text (36):
In summary, using the library approach described herein, optimal sequences of effective antisense compounds can be identified against specific mRNA targets. The antisense compounds are useful as potential therapeutics, as tools for drug target validation, in diagnostics and as a research reagent.

Detailed Description Text (7):
A chimaeric oligonucleotide library of the type described herein is incubated with an mRNA substrate. In this example, the target mRNA is TNF-.alpha. mRNA in a cellular extract. Incubation takes between one and three hours. As shown in step 1 of FIG. 6, two cut sites are produced at yields of 0.15% and 0.05% respectively. Most (99.8%) of the target mRNA is uncut. In this step, N and n each represent different nucleotides, as discussed above in the section on nucleotide chemistry. The library is either completely random (i.e. of the order of 10.sup.9 species) or specific to the target TNF-.alpha. (i.e. of the order of 1600 species).

Detailed Description Text (9):
In step 4, primers specific to the gene in question are designed to a region 5' of the poly A site. Using primers complementary to the gene and the dG (dC) tails, PCR is carried out to amplify full length target mRNA and cut target mRNA products.

Detailed Description Text (14):
Chimaeric oligonucleotide libraries were incubated with a target mRNA, in this case that of the TNF .alpha. gene.

Detailed Description Text (16):
Construction of Antisense Oligonucleotide Libraries

Detailed Description Text (17):
To determine the optimal length of antisense oligonucleotides required for specific mRNA hybridisation, oligonucleotide libraries of 8 nt and 12 nt have been synthesised by Oswel DNA Services Ltd. The libraries comprise of:

Detailed Description Text (32):
Cleavage of mRNA Using Gene Specific Libraries

Detailed Description Text (45):
6) Antisense oligonucleotide libraries were removed by spin column chromatography (Chromspin-30 DEPC treated, Clontech).

Detailed Description Text (48):
Cleavage of mRNA Using Random Libraries

Detailed Description Text (49):
The reaction protocol was as above for the gene specific libraries but using 20 .mu.M of the random library as supposed to 200 nM used above.

Detailed Description Text (51):
The oligonucleotide libraries were incubated with the in vitro transcribed TNFa mRNA and with RNAse H. To identify the sequences flanking the cut sites, the fragments generated in the incubation above were amplified by PCR using several primers targeted to various regions of the RNA thus ensuring that no combinations of cut fragments would be missed in the amplification steps.

Detailed Description Text (58):
The quantity of uncleaved mRNA is assayed by analysing separate overlapping regions of the target mRNA using PCR reactions such that the whole mRNA is covered by the overlapping regions. With this approach the worker does not need to know the binding site of the antisense ODN and therefore this method is ideal for use in the random library approach as well as for targeted antisense ODNs. Copies of the sequences corresponding to the overlapping regions that have been chosen are generated, with a reasonably sized specific internal deletion, e.g. about 50 bp, removed, such that they can be cloned to allow in vitro transcription of the deletion fragments. These deletion fragments can then be used as an internal standard RNA control for quantitative RT PCR.

Detailed Description Text (59):
A known amount of one of the standards is added to the RNA sample to be assayed. Following reverse transcription of this mixture, using a gene specific primer, PCR is carried out using fluorescently labeled primers and the reaction is run on an ABI 377 prism sequencing apparatus and the peaks corresponding to the amount of each product, is determined. Since the starting quantity of the standard is known, the amount of other products can be deduced by calibrating the intensity of the fluorescence of the products against that of the standard.

Detailed Description Text (90):
Primer Extenstion of Fragments from Series 1 of 12 nt Gene Specific Oligonucleotides

Detailed Description Text (97):
Primer Extenstion of Fragments from Series 1 of 8 nt Gene Specific Oligonucleotides

Detailed Description Text (104):
Primer Extenstion of Fragments from Series 2 of 12 nt Gene Specific Oligonucleotides

Detailed Description Text (111):
Primer Extenstion of Fragments from Series 2 of 8 nt Gene Specific Oligonucleotides

Detailed Description Text (116):
Primer Extenstion of Fragments from Series 3 of 12 nt Gene Specific Oligonucleotides

Detailed Description Text (118):
Unfortunately, no reliable data was acquired from these experiments or for series 38

mer libraries.

Other Reference Publication (6):
Larrouy et al., "RNase H-mediated inhibition of translation by antisense oligodeoxyribonucleotides: use of backbone modification to improve specificity", Gene, 1992, pp. 189-194, vol. 121, Elsevier Science Publishers B.V., Belgium.

Other Reference Publication (9):
Ho et al., "Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries", 1996, pp. 1901-1907, vol. 24, No. 1, Oxford University Press, Oxford, UK.

Other Reference Publication (10):
Studier, "A strategy for high-volume sequencing of cosmid DNAs: Random and directed priming with a library of oligonucleotides", Proc. Natl. Acad. Science, 1989, pp. 6917-6921, vol. 86, The National Academy of Sciences, Washington, D.C. USA.

CLAIMS:

1. A method of identifying an antisense binding site in a target mRNA, which comprises:

(1) an incubation step, wherein a target mRNA is incubated with an oligonucleotide library and a duplex-cutting RNAase under conditions which provide for the target mRNA to be cleaved at an antisense binding site; and

(2) an identification step, wherein the antisense binding site from the position of the cut in the mRNA is identified; wherein

(a) all of the oligonucleotides in the oligonucleotide library are present simultaneously in the incubation step with the target mRNA; and

(b) the oligonucleotide library comprises a plurality of distinct chimeric oligonucleotides capable of hybridizing to mRNA to form a duplex, the nucleotide sequences of which each have a common length ranging from 7 to 20 bases, which are generated randomly or generated based on the sequence of the target mRNA, wherein substantially all the nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are represented in the library, and wherein each nucleotide sequence comprises:

(a) a recognition region comprising a sequence of nucleotides that is recognized as a substrate by a duplex cutting RNAase when hybridized to the mRNA thereby permitting cleavage of said mRNA; and

(b) a flanking region on one or both sides of said recognition region, wherein said flanking region is distinct from said recognition region and comprises a sequence of chemically-modified nucleotides which binds to the mRNA sufficiently tightly to stabilize the duplex for cutting of the mRNA in the duplex by the duplex cutting RNAase, wherein the nucleotides constituting the flanking region are different from those constituting the recognition region, and wherein each oligonucleotide is protected against exonuclease attack.

2. A method according to claim 1, wherein the length of the nucleotide sequences present in the library ranges from 10 to 20 bases.